

# Gene Expression and Genetic Damage Indicators in Fish Exposed to Varying Stream Conditions in a Midwestern Watershed

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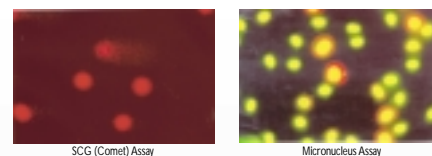
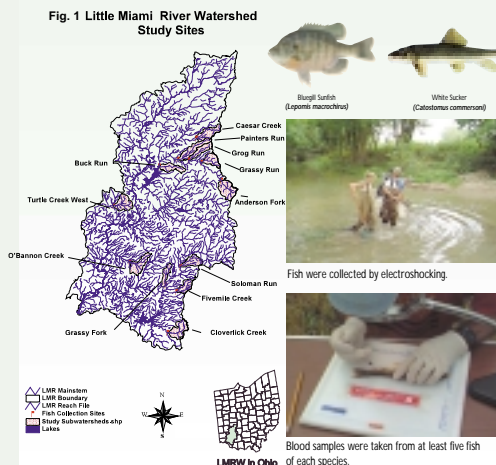
## Abstract:

Newly developed molecular diagnostic exposure indicators were evaluated as part of a greater study on the effectiveness of riparian zones to buffer streams from agricultural and urban environmental stressors. Water and fish samples collected from 12 headwater tributaries in the Little Miami River Basin were evaluated for the presence of estrogenic compounds and polycyclic aromatic hydrocarbons (measured by vitellogenin (Vg) and cytochrome P450A1 (P450) gene expression, respectively) and genotoxins (measured by single cell gel electrophoresis (SCG) and micronucleus (MN) assays). Larval fathead minnows (*Pimephales promelas*) were exposed to stream water and laboratory control water for 24 hrs. Total RNA was isolated and Vg and P450 gene expression were measured using designed synthetic oligonucleotide primers in quantitative RT-PCR. Fish blood samples obtained from bluegill sunfish (*Lepomis macrochirus*) and white suckers (*Catostomus commersoni*) were used for the SCG and MN analyses. For the SCG assay, the tail moment parameter was analyzed by computerized image analysis. Micronucleus frequencies were analyzed in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), and the PCE:NCE ratio determined. Results indicated an increase in P450 gene expression in fathead minnow larvae exposed to water samples from all 12 streams as compared to control laboratory water, the highest being observed in Turtle Creek. A marginal increase in Vg gene expression was observed only in Turtle Creek. Neither the SCG nor MN parameters from fish blood cells showed evidence of significant exposure to genotoxic contaminants in any of the streams. Relational databases for a range of field-measured stream condition parameters - nutrient levels and in-stream habitat measures - will be discussed.

## Introduction:

The study of the vulnerability of aquatic resources requires comparative exposure assessments across watersheds and regions which, in turn, require sensitive, diagnostic indicators of specific stressors. This investigation represents the first subregional survey of molecular and genetic damage indicators. The study was part of an overall evaluation of the effectiveness of riparian zones to buffer streams from agricultural and urban environmental stressors. The present pilot work was intended to assess the feasibility of the methodology and to evaluate the suitability of the fish species chosen (white suckers, sunfish and stonefishes) for monitoring environmental exposure to genotoxic contaminants. The specific aims were to: (1) compare the molecular indicators (Vg and P450) and the genetic damage indicators (SCG and MN) at various sites across the watershed and (2) compare these genetic indicators with water quality habitat and fish assemblage indicators.

Fig. 1 Little Miami River Watershed Study Sites



Methods:

### Fish and Water Collection:

Water and fish (by electro-shocking) were collected along 150 m sections of 12 headwater tributaries (third and fourth order streams) of the Little Miami River Watershed (Figure 1). All streams were set in a predominantly agricultural landscape but showed a range of land cover metrics, water chemistry conditions and in-stream habitat measures.

### Gene Expression:

**Test Water:** Stream water was tested within 48 hr after collection. Lab water was used as a negative control. 4 replicates/stream water. 400ml stream water/replicate.

**Test Species:** Fathead Minnow Larvae (*Pimephales promelas*) 24-48 hr old. 40 larvae/replicate.

**Test Duration:** 24 hr

**RNA Isolation:** Total RNA was isolated from 40 pooled larvae by the standard guanidinium isothiocyanate method.

**Reverse transcription of RNA followed by the Polymerase Chain Reaction (RT-PCR):** Vg-specific oligonucleotides used in RT-PCR amplification were from New England Biolabs and P450A1 fathead minnow-specific oligonucleotide mix were from Operon Technologies. Quantification of gene expression was accomplished with a multiplex PCR reaction using the specific oligonucleotides and Competitor/18S ribosomal RNA oligonucleotides (Ambion).

**Verification of PCR Products:** 1.8% Agarose gels were used for the electrophoresis of the amplification products. Gels were stained with SyberGreen I (Molecular Probes), digitally scanned using a FluorImager 595 system and relative band intensities for each Vg and P450 gene and 18S Competitor were analyzed with ImageQuant software.

### Genetic Damage Indicators:

#### Target Species

- Sunfish species (*Lepomis* sp.)
- White suckers (*Catostomus commersoni*)

#### Collection of Blood Samples:

Blood samples were taken from at least five fish per site of each species. Blood samples were drawn from caudal vein into heparinized syringe; 5 µl were used to prepare duplicate blood smears for MN assay. The remainder was kept on ice and returned to lab for use in SCG assay

#### SCG Assay Endpoints:

**Tail Moment:** (Tail length x % Tail DNA)/100. Analyzed by a Komet computerized image analysis system

#### MN Assay Endpoints:

- Ratio of PCE (polychromatic erythrocytes) to NCE (normochromatic erythrocytes)
- MN frequency in PCEs and NCEs

### Statistical Analysis:

- One-way ANCOVA among treatment groups
- Spearman Rank Correlation
- Dunnnett's Group Mean Comparison

## Results:

The results of gene expression analyses indicated an overall increase in P450 gene expression in fathead minnow larvae exposed to water samples for 24 hrs from all 12 streams as compared to control laboratory water (Fig 2 and 4B). A significant induction of the P450 gene expression was seen in Turtle Creek, Soloman Run, Anderson Fork and Buck Run. In fact, Turtle Creek showed the highest P450 transcription level which was 360% induction over the control water. As for the Vg gene expression, only the water samples collected from Turtle Creek showed a significant increase in Vg mRNA (Fig 3 and 4A). On the contrary, a significant reduction in Vg gene expression in fathead minnow larvae was observed for the water samples collected from Caesar Creek and Grassy Run sites as compared to a control lab water.

Results of the MN assay showed the mean  $\pm$  sem % PCE levels were  $7.80 \pm 0.78$  (n = 45) for sunfish and  $8.60 \pm 0.78$  (n = 35) for white suckers. The mean  $\pm$  sem MN frequencies (MN/1000 NCEs) were  $0.033 \pm 0.019$  for sunfish and  $0.016 \pm 0.004$  for white suckers. The MN frequencies and the % PCE results are plotted in Fig 5A and 5B. The MN frequencies for sunfish were very low, comparable to laboratory control fish (data not shown). Results for sunfish were obviously lower than for white suckers. Differences in MN levels between sunfish and white suckers might be due to differing exposures of these two species to contaminants in the water column and sediment, respectively. The % PCE frequencies did not differ significantly between species. % PCE did not vary significantly across sampling sites for sunfish, but for white suckers, % PCE were significantly higher in Cloverlick Cr. than for O'Bannon Cr.

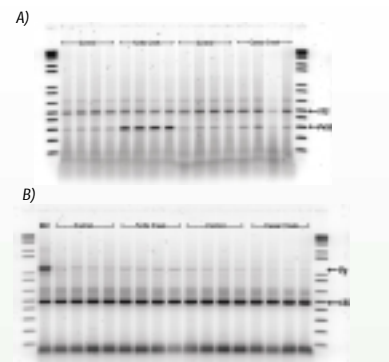


Fig. 2 Agarose gel image of RT-PCR amplification products of (A) P450 and (B) Vitellogenin and internal Competitor/18S standard in fathead minnow larvae exposed to site water for 24 hr.

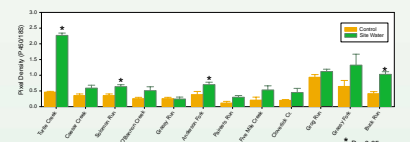


Fig. 3 P450 gene expression in fathead minnow larvae exposed to site water and control laboratory water.

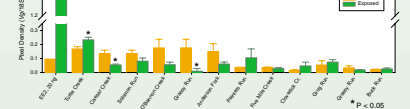


Fig. 4 Vitellogenin gene expression in fathead minnow larvae exposed to site water and control laboratory water.

- The SCG parameter, tail moment, results are shown in Fig 6 and 7, for sunfish and white sucker, respectively. The mean  $\pm$  sem tail moment (TM) were  $2.16 \pm 0.14$  (n = 46) for sunfish and  $2.69 \pm 0.15$  (n = 46) for white sucker. The values for sunfish were comparable to laboratory control values, and did not vary significantly among sites for either species. No significant differences were observed among sites for white suckers. Differences were observed among sites for sunfish-Turtle Creek was higher than Grassy Run or Grog Run.
- Several other stream condition parameters, including index of Biotic Integrity (IBI) scores and nutrient levels are shown in Fig 8. The nutrient levels showed substantial variability from site to site. Negative correlations were observed between the total nitrogen and the comet parameter for both white suckers ( $p=0.64$ ) and sunfish ( $p=0.57$ ). A positive correlation was observed between total phosphorus and the comet parameters in white suckers ( $p=0.75$ ). A high negative correlation was also observed between % PCE and IBI values for white suckers ( $p=0.88$ ), but not for sunfish.

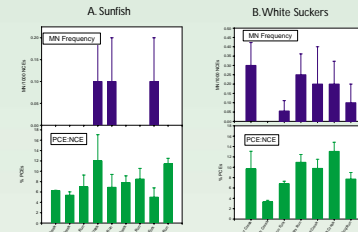


Fig. 5 MN frequency and PCE:NCE ratio in fish erythrocytes. (A) Sunfish and (B) White suckers.

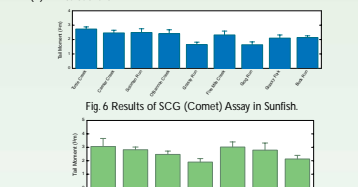


Fig. 6 Results of SCG (Comet) Assay in Sunfish.

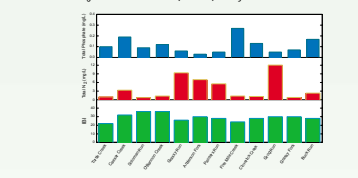


Fig. 7 Results of SCG (Comet) Assay in White Sucker.

Fig. 8 Total phosphate (mg/L), total nitrogen (mg/L) and index of biotic integrity (IBI) in the Little Miami River watershed in August 2000.

## Discussion and Conclusions:

Measurement of gene expression levels by RT-PCR is a sensitive method for detecting exposure to environmentally relevant concentrations of chemicals. We have applied this method to the vitellogenin gene as an indicator of exposure to endocrine disrupting compounds and the cytochrome P450A1 gene for exposure to polycyclic aromatic hydrocarbons. The levels of Vg gene expression in fathead minnow larvae exposed to the water collected from the study sites were very low, and most below the level of the control laboratory water. A notable exception was Turtle Creek water which gave a significant induction in Vg. However, the P450 gene expression in fathead minnow larvae were induced by all water samples collected; in particular, a high level was observed in Turtle Creek. Turtle Creek was located next to a golf course and fertilizers and pesticides used in the golf course might contribute to the high induction of P450 gene expression.

The levels of genetic damage in fish collected from the study sites were very low, within the range expected for background levels of damage. These findings were not unexpected since the sites selected were not expected to be heavily impacted by contaminants. Although some statistical correlations were observed between the genetic damage parameters and other stream condition parameters, the biological significance of these correlations is unclear, and awaits confirmation in further studies. Future efforts will include study sites with a significant agricultural gradient, as well as sites representing a wider range of contamination. This will be important in establishing the sensitivity of the methods and for evaluating their ultimate utility for watershed and regional evaluations.